APPENDIX C (cont.)

An element-by-element comparison of the Mehlhorn claims with the Cramer Reference

Cramer is § 102(b) prior art as of its 1977 publication date. This reference discloses that carboxylic acids can be loaded into liposomes via a mechanism analogous to the mechanism used by Nichols to load amines. In other words, the loading of carboxylic acids into liposomes with a basic internal aqueous phase by the addition of an acid to the external medium as taught by Cramer is the flip side of Nichols. Indeed, Cramer makes this very point at the outset by expressly reciting to Nichols in note 27:

> Thus, lowering the pH of a carboxylic acid solution, which is external to a closed vesicular membrane, should concentrate the acid in the interior volume of the vesicle. **An analogous mechanism** has been proposed for the concentration of catecholamines within chromaffin vesicles (1) and **has led to recent studies of the correlation of pH gradients with transport phenomena** [27].

FR Exh 5, Cramer at pp. 295-96 (emphasis added).

Specifically, Cramer discloses the formation of liposomes from egg phosphatidylcholine and cholesterol in an aqueous solution of maleate buffer as called for by step (a)(ii) of Mehlhorn claims 27 and 38. A deuterated water solution containing fumarate buffer (mostly in an anionic form) was then added to the external liposome phase. This meets step (b)(ii) of claims 27 and 38, adding a charged anionic species to the external liposome phase. This step was followed by the addition of DCl (deuterated hydrochloric acid) to the external medium to form a pH gradient to induce neutral fumaric acid to pass through the lipid bilayer into the internal aqueous compartment, which meets step (c)(ii) of Mehlhorn claims 27 and 38. (See Prestegard Declaration, at ¶ 11, FR vol II, tab 8 at 17). Cramer perform steps (a) through (c) in that order,

i.e., adding the chemical species of step (b) prior to forming the pH gradient of step (c) in the same manner the claimed steps are performed in Mehlhorn.

The details of the comparison are set forth in the Claim Chart II, focusing on subpart (ii), below:

Claim Chart II

Mehlhorn	Cramer
27. A method of preparing a liposome vesicle-entrapped charged chemical species which comprises:	Cramer teaches a method for preparing liposomes containing fumarate [INTRODUCTION, p. 296, lines 6-8; RESULTS, p. 297, line 1 to p. 298, line 3] by:
(a) forming liposomes in: (ii) an aqueous medium containing a base which is substantially impermeable through the vesicle	forming vesicles (liposomes from egg phosphatidyl-choline and cholesterol in an aqueous solution containing maleate (0.19M maleic acid at pH 7) [MATERIALS & METHODS, p. 296, lines 1-3] which is "substantially impermeable" through the vesicle [INTRODUCTION, p. 295, lines 6-8; DISCUSSION, p. 299, lines 2-4] (as evidenced by establishment of a pH gradient upon addition of deuterated hydrochloric acid to the external liposome phase [MATERIALS & METHODS, p. 297, lines 6-7; March 22, 1993 Amendment, Paper No. 9, p. 10, lines 7-17])
to give a basic liposome-containing aqueous medium in which the base is present in the internal and external liposome phases;	to give a basic aqueous medium containing liposomes; maleate (a weak base) was present in both the internal and external liposome phases [MATERIALS & METHODS, p. 296, lines 1-3];

(b) adding: (ii) to the thus-obtained basic liposome-containing aqueous medium a charged chemical species which is anionic, and	adding fumarate to the external liposome phase by exchanging the external maleate solution with an equiosmolar solution containing fumarate [MATERIALS & METHODS, p. 296, line 4 to p. 297, line 2];
(c) adding to the external liposome phase: (ii) an acid to thereby induce the anionic chemical species to pass into the liposomes' internal basic aqueous phase.	adding 0.4N deuterated hydrochloric acid to the external liposome phase to establish a transmembrane pH gradient, which induced accumulation of fumarate inside the vesicles [MATERIALS & METHODS, p. 297, lines 6-7; RESULTS, p. 297, lines 2-4].
29. The method of Claim 27, wherein in (a) (ii) a basic liposome-containing aqueous medium is formed in which the base is present in both the internal and external liposome phases, and in (c) (ii) an acid is added to the external phase to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase.	Cramer teaches forming vesicles from egg PC and cholesterol in an aqueous maleate solution; the maleate is present in both the internal and external liposome phases [MATERIALS & METHODS, p. 296, lines 1-3 and p. 297, lines 1-5]; and adding deuterated hydrochloric acid to the external liposome phase to cause fumarate to pass into the vesicles' internal aqueous phase [MATERIALS & METHODS, p. 297, lines 6-7; RESULTS, p. 297, lines 2-4].
30. The method of Claim 27, wherein the aqueous medium containing . . . the base used in forming the liposomes in (a) (ii), is buffered.	Cramer teaches using an aqueous maleate solution (0.19M maleic acid at pH 7) as the aqueous medium for forming the liposomes [MATERIALS & METHODS, p. 296, lines 1-4]; the aqueous maleate solution is buffered, i.e., contains the conjugate acid (maleic acid) of the base (maleate).
31. The method of Claim 27, wherein the . . . acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c) (ii) is a component of a buffer.	Cramer teaches adding deuterated hydrochloric acid to establish a pH gradient and thereby induce accumulation of fumarate in the vesicles' internal aqueous phase [MATERIALS & METHODS, p. 297, lines 6-7], the deuterated hydrochloric acid raises <u>in situ</u> the acidic components of a buffer, i.e., the external aqueous phase contains more fumaric acid (weak acid).

32. The method of Claim 30 wherein the . . . acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c) (ii), is a component of a buffer.	Cramer teaches adding deuterated hydrochloric acid to establish a pH gradient and thereby induce accumulation of fumarate in the vesicles' internal aqueous phase [MATERIALS & METHODS, p. 297, lines 6-7], the deuterated hydrochloric acid raises <u>in situ</u> the acidic components of a buffer, i.e., the external aqueous phase contains more fumaric acid (weak acid).
38. A method of preparing a liposome entrapped charged chemical species which comprises:	Cramer teaches a method for preparing liposomes containing fumarate [INTRODUCTION, p. 296, lines 6-8; RESULTS, p. 297, line 1 to p. 298, line 3] by:
(a) forming liposomes in: (ii) an aqueous medium containing a base	forming vesicles (liposomes) from egg phosphatidyl-choline and cholesterol in an aqueous solution containing maleate (0.19M maleic acid at pH 7) [MATERIALS & METHODS, p. 296, lines 1-3]
which is substantially impermeable through the vesicle	which is "substantially impermeable" through the vesicle [INTRODUCTION, p. 295, lines 6-8; DISCUSSION, p. 299, lines 2-4] (as evidenced by establishment of a pH gradient upon addition of deuterated hydrochloric acid to the external liposome phase [MATERIALS & METHODS, p. 297, lines 6-7; March 22, 1993 Amendment, Paper No. 9, p. 10, lines 7-17])
to give a basic liposome-containing aqueous medium in which the base is present in the internal and external liposome phases;	to give a basic aqueous medium containing liposomes; maleate (a weak base) was present in both the internal and external liposome phases [MATERIALS & METHODS, p. 296, lines 1-3];

(b) adding: (ii) to the thus-obtained basic liposome-containing aqueous medium a charged chemical species which is anionic, and	adding fumarate to the external liposome phase by exchanging the external maleate solution with an equiosmolar solution containing fumarate [MATERIALS & METHODS, p. 296, lines 4 to p. 297, line 2];
(c) adding to the external liposome phase: (ii) an acid in an amount effective to create a pH gradient between the external liposome phase and the internal liposome phase to thereby induce the anionic chemical species to pass into the liposomes' internal basic aqueous phase.	adding 0.4N deuterated hydrochloric acid to the external liposome phase to establish a transmembrane pH gradient, which induced accumulation of fumarate inside the vesicles [MATERIALS & METHODS, p. 297, lines 6-7; RESULTS, p. 297, lines 2-4].
40. The method of Claim 38, wherein in (a) (ii) a basic liposome-containing aqueous medium is formed in which the base is present in both the internal and external liposome phases, and in (c) (ii) an acid is added to the external phase to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase..	Cramer teaches forming vesicles from egg PC and cholesterol in an aqueous maleate solution; the maleate is present in both the internal and external liposome phases [MATERIALS & METHODS, p. 296, lines 1-3 and p. 297, lines 1-5]; and adding deuterated hydrochloric acid to the external liposome phase to cause fumerate to pass into the vesicles' internal aqueous phase [MATERIALS & METHODS, p. 297, lines 6-7; RESULTS, p. 297, lines 2-4].
41. The method of Claim 38 wherein the aqueous medium containing the . . . base used in forming the liposomes in (a) (ii), is buffered.	Cramer teaches using an aqueous maleate solution (0.19M maleic acid at pH 7) as the aqueous medium for forming the liposomes [MATERIALS & METHODS, p. 296, lines 1-4]; the aqueous maleate solution is buffered, i.e., contains the conjugate acid (maleic acid) of the base (maleate).

42. The method of Claim 38, wherein the . . . acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c) (ii), is a component of a buffer.

Cramer teaches adding deuterated hydrochloric acid to establish a pH gradient and thereby induce accumulation of fumarate in the vesicles' internal aqueous phase [MATERIALS & METHODS, p. 297, lines 6-7], the deuterated hydrochloric acid raises in situ the acidic components of a buffer, i.e., the external aqueous phase contains more fumaric acid (weak acid).

43. The method of Claim 41 wherein the . . . acid which is added to thereby induce the anionic chemical species to pass into the liposomes' internal aqueous phase in (c) (ii), is a component of a buffer.

Cramer teaches adding deuterated hydrochloric acid to establish a pH gradient and thereby induce accumulation of fumarate in the vesicles' internal aqueous phase [MATERIALS & METHODS, p. 297, lines 6-7], the deuterated hydrochloric acid raises in situ the acidic components of a buffer, i.e., the external aqueous phase contains more fumaric acid (weak acid).